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Temperature-Sensitive Mutants of *Actinobacillus pleuropneumoniae* Induce Protection in Mice

WYATT BYRD AND ANNE MORRIS HOOKE*

Department of Microbiology, Miami University, Oxford, Ohio 45056

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Temperature-sensitive mutants of *Actinobacillus pleuropneumoniae* 4074, serotype 1, were isolated after treatment with nitrosoguanidine and enrichment with penicillin and D-cycloserine. Of the four temperature-sensitive mutants evaluated in mice, one (A-1) had a tight phenotype (i.e., it ceased replication immediately after transfer to the nonpermissive temperature [37°C]) and three (1-2, 4-1, and 12-1) were coasters that continued replication for up to three generations after transfer to 37°C. The reversion frequencies ranged from 10^{-6} to 10^{-9}, and cutoff temperatures ranged from 33 to 35°C. No major changes were detected in the biochemical profiles; agglutination reactions; electrophoretic profiles of the lipopolysaccharides, outer membrane proteins, and hemolysin proteins; hemolytic titers; or CAMP factor reactions of the mutants and the wild-type bacteria. Groups of 3- to 5-week-old, female ICR mice were immunized intranasally with three doses of 3.5×10^6 CFU of the mutants over 3 weeks and subsequently challenged intranasally with 5 50% lethal doses of the parental wild-type. Protection was induced by both the tight and the coaster mutants, with the 4-1 and 12-1 coasters eliciting greater protection (67 and 82%, respectively) than that induced by the A-1 tight mutant (57%). Intranasal immunization with both phenotypes induced serum antibody responses against the surface antigens and the hemolysin protein.

Swine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* (SPAP) is a respiratory infection of swine (28) that ranges from an acute phase, with high mortality, to the development of chronic lung lesions. Surviving pigs suffer reduced growth rates and frequently become asymptomatic carriers of the organism (27).

The capsular polysaccharide (CP) (16), lipopolysaccharide (LPS) (30), outer membrane proteins (OMP) (5), and hemolysin protein (HP) (15) contribute to the pathogenesis of swine pleuropneumonia. Immunization of swine with either heat-killed or formalin-treated whole cells induces only a limited, serotype-specific protection (13, 26). Pigs that survive the natural infection, however, are often protected against reinfection with *A. pleuropneumoniae* of any serotype (25).

We are investigating the feasibility of using temperature-sensitive (TS) mutants to develop a vaccine to prevent SPAP. We isolated two different TS phenotypes of *A. pleuropneumoniae*: both replicate normally at 30°C, but at 37°C they either completely cease replication (tight mutants) or continue growth for two to three generations (coaster mutants). The growth characteristics, reversion frequencies, and biochemical and physical profiles of these TS mutants were determined, and the immunogenicity of the TS mutants was evaluated in mice.

MATERIALS AND METHODS

Bacteria and growth media. *A. pleuropneumoniae* 4074, serotype 1, was obtained from the American Type Culture Collection (Rockville, Md.) and stored in Trypticase soy broth (TSB)–7% dimethyl sulfoxide at −70°C; the TS mutants were similarly stored. The bacteria were cultured in TSB with 0.6% yeast extract and 0.01% NAD (TSB-NAD). Hemolysin protein was purified from TS and wild-type (WT) strains cultured in chemically defined medium (CDM) (20).

Isolation of TS mutants. Standard methods of chemical mutagenesis and enrichment were used to isolate the TS mutants (23). *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (3 μg/ml) was added to a log-phase culture of the WT bacteria in TSB-NAD, and the bacteria were incubated for 10 min at 37°C without aeration. The cells were washed (three times) free of mutagen, diluted, and incubated overnight at the permissive temperature (30°C) to allow segregation of the mutations. The log-phase cultures were then shifted to the nonpermissive temperature (37°C) for one round of replication; penicillin G (100 U/ml) was added, and the cultures were incubated at 37°C for 2 h, allowing the enrichment of the TS mutants, as only those cells able to divide at 37°C were killed by the penicillin and, by delaying the addition of the penicillin for one generation, enrichment for TS mutants of the coasting phenotype was enhanced. After 2 h, the cells were washed (three times) free of the penicillin, suspended in fresh TSB-NAD, and incubated at 30°C for several hours. The log-phase cultures at 30°C were again shifted to 37°C and incubated for 1 to 2 h. The second cycle of enrichment with a different cell wall antibiotic, D-cycloserine (30 mM), was carried out at 37°C for 2 h. The survivors were washed (three times) free of the D-cycloserine, diluted appropriately, plated, and incubated at 30°C. Those colonies that appeared at 30°C were replica plated at 30°C and 37°C, and the TS mutants were identified and subcultured for characterization.

Characterization of TS mutants. The TS mutants were characterized with respect to their growth phenotypes and reversion frequencies. For the initial screening, the TS mutants were streaked on several plates and incubated at 23, 29, 31, 33, or 37°C for 24 to 48 h, and the presence or absence of colonies at each temperature was noted.

Growth profiles of the TS mutants in broth were determined by splitting a 30°C log-phase culture and incubating one part at 30°C and the other at 37°C. Growth at the two temperatures was monitored spectrophotometrically (at 600 nm) and by viable cell counts. Revertant frequencies of the TS mutants were determined by plating 10^7 to 10^9 cells and incubating them at 37°C for 48 h. Coasting mutants were held at the nonpermissive temperature until they ceased all replication before plating. The revertant frequency was calculated as the ratio of CFU at 37°C to the number of viable cells plated (determined by incubating appropriate dilutions at 30°C).

Biochemical and physical evaluation of TS mutants. Biochemical tests, agglutination, LPS and OMP profiles, HP production, and CAMP factor reactions were used to detect any phenotype alterations in the TS mutants.

Biochemical profiles were determined with the Sceptor automated identification system (Becton Dickinson, Towson, Md.) according to the manufacturer's instructions, except that the results were recorded after incubation at 30°C for 24 h.

Agglutination was measured by adding 500 μl of the TS mutants or WT bacteria (approximately 10^8 CFU) to 500 μl of 1:2-diluted immune serum, preimmune mouse serum, or saline on a ringed-glass slide. The slides were incubated at room temperature for 10 min with rocking, and agglutination was recorded.

The LPS, OMP, and HP profiles were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The LPS gels were silver stained (Bio-Rad, Hercules, Calif.), and hemolysin and OMP gels were stained with Coomassie blue (R-250; Bio-Rad).

Hemolytic activity. The hemolytic activity of the TS mutants was determined qualitatively with sheep erythrocytes (RBC). Single colonies from a TSB-NAD

* Corresponding author. Phone: (513) 529-2028. Fax: (513) 529-2431. E-mail: AMH@MUOHIO.EDU.

plate were streaked on two 5% sheep blood agar plates containing 0.01% NAD (BAP-NAD). One plate was incubated at 30°C, and the other was incubated at 37°C. After 24 and 48 h of incubation the hemolysis surrounding the streaks on both plates was noted.

Supernatants from WT bacteria and TS mutants incubated at 30 and 37°C were quantitatively assayed for hemolytic activity as previously described (29). Briefly, bovine RBC (2%) in 0.15 M NaCl were mixed with an equal volume of supernatants from either 30 or 37°C cultures serially diluted (twofold) in 20 mM Tris-HCl, 5 mM $CaCl_2$, and 150 mM NaCl, pH 7.8. The mixtures were incubated for 60 min at 37°C and then centrifuged for 1 min at 8,000 × *g*, and the absorbance of the supernatants was measured at 545 nm. One hemolytic unit was defined as the activity in the dilution that caused 50% lysis of the RBC compared with 100% lysis of 0.25 ml of RBC suspension. The titer in hemolytic units per milliliter was expressed as the reciprocal of the dilution corresponding to 50% lysis.

The CAMP factor assay was done as described by Kilian (18). Briefly, *Staphylococcus aureus* was streaked across a 5% sheep BAP-NAD; the WT and TS mutant strains were streaked at right angles to the *S. aureus*, and CAMP activity was noted as an increase of the hemolytic zones after incubation of plates at 30 and 37°C.

Preparation of bacterial antigens. The procedure of Adlam et al. (1) with modifications (2) was used to isolate and purify the CP from *A. pleuropneumoniae*. Briefly, CP was released from the surface of the bacteria by vigorous agitation, and the supernatant was filtered. The CP was precipitated with cold acetone, the mixture was centrifuged, and the pellet was suspended in Tris buffer and treated with DNase (200 μg/ml), RNase (50 μg/ml), and proteinase (200 μg/ml). Cold acetone was again added, the mixture was centrifuged, and the pellet was dissolved and extracted three times in 10% saturated sodium acetate–77% (wt/vol) phenol; the aqueous phases were dialyzed against 0.1 M $CaCl_2$. Three volumes of ethanol were added, the mixture was centrifuged, and the ethanol-purified CP was lyophilized. The CP preparation was essentially free of nucleic acid and protein (less than 0.1% [wt/wt]) and LPS (less than 0.01% [wt/wt]).

The procedure of Darveau and Hancock (4) was used to purify the LPS from *A. pleuropneumoniae*. Bacteria disrupted by a French pressure cell (SLM Instruments, Urbana, Ill.) were treated with DNase (1,400 μg/ml), RNase (375 μg/ml), proteinase (200 μg/ml), and SDS, and the LPS extract was subjected to 95% ethanol–0.375 M magnesium chloride precipitation, high-speed centrifugation, and lyophilization. The preparation was essentially free (less than 0.1% [wt/wt]) of nucleic acid and protein.

Outer membrane proteins were isolated by a modified method of Fedorka-Cray et al. (8). Cultures were incubated in TSB-NAD at 30°C until the A_{600} was 0.4 to 0.5, or the TS mutants were treated as described above until the A_{600} was 0.2 to 0.3, when the temperature was increased to 37°C and incubation was continued for 3 h. The cultures were centrifuged at 13,000 × *g* for 20 min, and the pellets were suspended in 1 ml of 10 mM HEPES, pH 7.4. Cells were disrupted with a sonicator (model 300; Fisher Scientific, Fair Lawn, N.J.) at 75% maximum output. The suspensions were sonicated for six 1-min bursts and then centrifuged at 8,800 × *g* for 10 min, and the membrane fractions were collected by centrifuging the supernatant at 18,000 × *g* for 30 min. Pellets were suspended in 0.2 ml of 10 mM HEPES, and an equal volume of 2% Sarkosyl (sodium-*N*-lauroyl sarcosine) (Fisher Scientific) in 10 mM HEPES was added. The sample was incubated at room temperature for 30 min with gentle shaking and centrifuged at 18,000 × *g* for 30 min. The Sarkosyl-insoluble OMP pellet was washed once in 10 mM HEPES without vortexing and then centrifuged at 18,000 × *g* for 5 min. The pellet was suspended in 100 μl of 10 mM HEPES and stored at −70°C. The OMP preparation contained approximately 10^5 endotoxin units (EU)/ml.

Hemolysin protein was purified from *A. pleuropneumoniae* cultured in CDM (20) at either 30 or 37°C until mid- to late log phase. The culture was centrifuged at 27,000 × *g* for 20 min at 4°C, and the supernatant was filtered. The pH of the cell-free supernatant was adjusted to 6.2, ammonium sulfate (85% saturation) was added, and the suspension was stirred overnight at 4°C. The resultant precipitate was collected by centrifugation at 27,000 × *g* for 30 min at 4°C. The pellet was suspended in 3 ml of 50 mM phosphate-buffered saline (PBS) (pH 7.2) and dialyzed against 50 mM PBS (pH 7.2) at 4°C. The HP preparation contained approximately 10^3 EU/ml.

Chemical analysis. The CP, LPS, OMP, and HP preparations were analyzed for protein, carbohydrate, and/or nucleic acid content. Total protein was determined by the bicinchoninic acid colorimetric microtiter assay (Pierce, Rockford, Ill.), with bovine serum albumin as the protein standard. The carbohydrate content was assayed by the phenol-sulfuric acid procedure (6), with glucose as the carbohydrate standard. Nucleic acid content was determined by measuring the absorbance at 260 and 280 nm.

LPS assay. Endotoxin in the CP, OMP, and HP preparations was determined by the colorimetric *Limulus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, Md.) for the rapid chromogenic quantitation of bacterial endotoxin. Purified *Escherichia coli* LPS was used as the standard (2 ng/EU).

SDS-PAGE. SDS-PAGE used the Laemmli buffer system (19): the separating gel of 12% (wt/vol) acrylamide and bisacrylamide in 0.375 M Tris-HCl, pH 8.8, and a stacking gel of 4% (wt/vol) acrylamide and bisacrylamide in 0.125 M Tris-HCl, pH 6.8. The purified preparations were diluted (1:2) into sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol), heated to 95°C for 5 min, and subjected to electrophoresis at a constant current of 40 mA. The LPS gels were silver stained, and the OMP and hemolysin gels were Coomassie blue (R-250) stained.

Immunoprotection studies. The TS mutants were evaluated for their potential to induce protection in mice (3 to 5 weeks old; ICR; female) from challenge with WT *A. pleuropneumoniae*. Log-phase cells grown at 30°C were washed twice with PBS and suspended in PBS, and approximately 3.5×10^6 CFU in 50 μl was administered intranasally (i.n.) to each mouse; 10 and 17 days later second and third doses of 3.5×10^6 CFU were given, and 15 days after the final dose the mice were challenged i.n. with 5 50% lethal doses (LD_{50}) (1×10^7 CFU) of the WT bacteria. A group of control mice inoculated i.n. with PBS was also challenged. The mice were monitored daily for morbidity and mortality until day 14 after challenge, when all surviving mice were sacrificed and necropsied.

Antibody titers. Antibodies against the CP, LPS, OMP, and HP of *A. pleuropneumoniae* were measured by an enzyme-linked immunosorbent assay (ELISA) (3). Blood samples were collected from the tail veins 14 days after the third dose and 14 days after challenge. Twofold serially diluted sera were added to antigen-coated wells, followed by goat anti-mouse polyvalent immunoglobulin G (IgG), IgA, and IgM conjugated with alkaline phosphatase (Sigma Chemical, St. Louis, Mo.). Absorbance was read at 410 nm on an ELISA reader (Minireader II; Dynatech Laboratories, Alexandria, Va.). Titers were defined as the reciprocal of the highest dilution of test immune serum at which the A_{410} was at least twice that of the nonimmune serum at the same dilution.

The sera were treated as previously described (17) to remove antibodies to the LPS. Briefly, 1 ml of serum was mixed with 5 mg of LPS isolated from *A. pleuropneumoniae* and 0.5% sodium azide. The mixture was gently rocked overnight at 4°C and then centrifuged at 105,000 × *g* for 4 h at 4°C, and the absorbed serum was stored at −70°C until assayed for antibodies against the OMP and HP. The absorbed sera were unreactive in ELISAs with LPS as the antigen (data not shown).

Statistical analysis. Death rates of the TS mutant-vaccinated and unvaccinated challenged mice were compared with the Bonferroni multiple-comparison test. Student's *t* test for independent means was used to calculate *P* values for antibody responses. The level of significance for all statistical analyses was $P < 0.05$.

RESULTS

Isolation and characterization of TS mutants. More than 50 TS mutants were isolated, and after preliminary characterization of phenotype, reversion frequency, and OMP profiles, 4 were chosen for more-detailed analysis. The growth characteristics of four TS mutants, 1-2, 4-1, 12-1, and A-1, were determined on solid media at different temperatures and in liquid culture at 30 and 37°C. The growth profiles of two TS mutants (one tight and one coaster) after transfer to the nonpermissive temperature are shown in Fig. 1. Three mutants continued replication for two to three generations after transfer to the nonpermissive temperature, whereas the A-1 mutant ceased replication immediately after transfer to 37°C. The TS mutants maintained full viability even after incubation at 37°C for 11 h (data not shown). All the mutants grew on TSA-NAD at temperatures from 23 to 31°C. The growth of the tight mutant and two of the coasters ceased at 33°C, and that of the third coaster ceased at 35°C (Table 1). The revertant frequencies of the four mutants ranged from 2×10^{-6} to 7×10^{-9} (Table 1).

Biochemical and physical comparisons. The biochemical profiles and agglutination reactions of the TS mutants were identical to those of the WT (data not shown). Analysis of LPS (data not shown), OMP (Fig. 2), and HP (Fig. 3) by SDS-PAGE failed to detect any major differences between the TS mutants and the WT bacteria. The TS mutants and the WT were all beta-hemolytic on sheep BAP-NAD at 30°C. Quantitative hemolytic titers of culture supernatants from both mutants and WT grown at 30°C were essentially identical: 32 to 64. Quantitative hemolytic activity could not be detected in the supernatants of mutants inoculated from a 30°C plate into CDM and held at 37°C for several hours. Both the mutants and the WT gave identical CAMP reactions on sheep BAP-NAD incubated at 30°C; at 37°C, the CAMP reaction was negative for all the TS mutants (because they do not grow at that temperature) and positive for the WT.

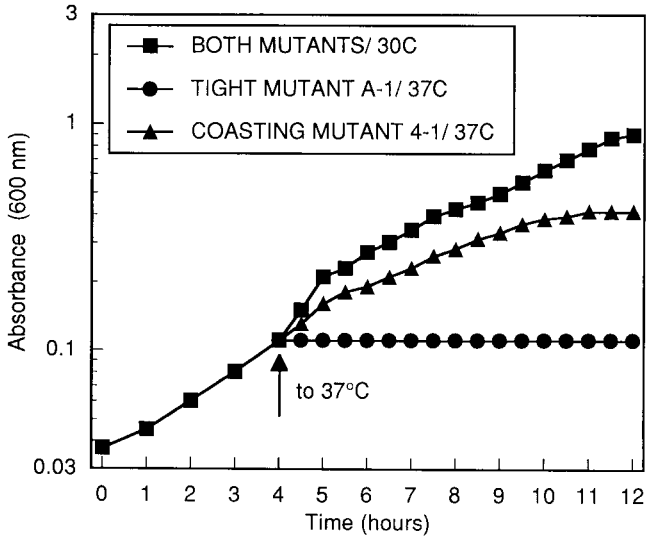

FIG. 1. Growth profiles of the tight (A-1) and coaster (4-1) TS mutants of *A. pleuropneumoniae* 4074 at 30 and 37°C. The TS mutants were cultured in TSB with NAD at 30°C. At the time indicated by the arrow, the cultures were divided; half were incubated at 30°C, and the others were incubated at 37°C.

Immunoprotection. Two strains were tested in preliminary immunoprotection studies in mice: A-1, a tight mutant, and 1-2, a coaster. No protection was induced by a single i.n. dose of 3.5×10^6 CFU of either the A-1 or 1-2 TS mutant (0 and 17% survival, respectively). Subsequently, two doses were administered after the initial immunizing dose, and the protection induced by A-1 (57% of mice survived challenge) was significant ($P = 0.04$). The increased protection in mice immunized three times with 1-2 (38%) was not significant, so we did not consider it any further.

We then immunized mice with three doses of the A-1 tight mutant and the 4-1 and 12-1 coaster mutants. The 12-1 TS mutant induced the best protection, with 82% of the mice surviving challenge; the 4-1 coaster induced some protection, with 67% of the mice surviving challenge; and the A-1 tight mutant induced the least protection (57% survival) (Table 2). The challenge dose (5 LD_{50}) caused distress in all the mice, but most of the immunized mice recovered within 48 h. By 72 h, all 17 mice in the control group had died.

The ELISA titers of sera from immunized and challenged mice to the CP, LPS, OMP, and HP of *A. pleuropneumoniae* are given in Table 3; i.n. immunization of mice with all mutants tested induced serum antibody responses against the polysaccharide and protein components of *A. pleuropneumoniae*. Immunization with the 4-1 coaster mutants induced significantly

TABLE 1. Characteristics of TS mutants of *A. pleuropneumoniae*

Mutant	Phenotype	Doublings at 37°C[a]	Cutoff temp (°C)[b]	Revertant frequency[c]
A-1	Tight	0	33	8×10^{-8}
1-2	Coaster	3	35	7×10^{-9}
4-1	Coaster	2	33	4×10^{-7}
12-1	Coaster	2	33	2×10^{-6}

[a] Cultures were incubated at 30°C until mid-log phase and then shifted to 37°C. Growth was monitored by measuring the absorbance at 600 nm.

[b] Cultures were spread on plates and incubated at different temperatures, and growth at each was recorded.

[c] Cultures incubated at 30°C were plated at high densities ($>10^6$ CFU per ml) and incubated at 37°C.

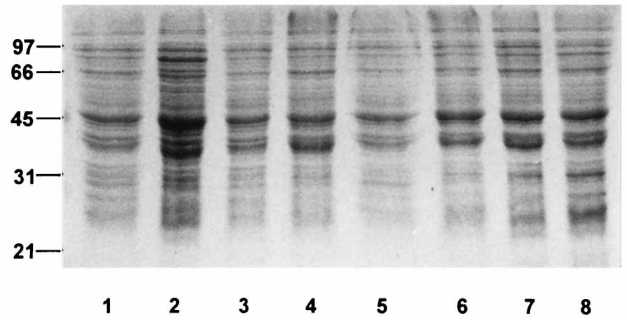

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel of OMP from the WT and TS mutants of *A. pleuropneumoniae* 4074. The OMP (8 to 10 μg of protein per lane) were prepared, as described in Materials and Methods, from bacteria grown at 30°C for the entire incubation (lanes 1, 3, 5, and 7) and from bacteria grown at 30°C until mid-log phase, when the temperature was increased to 37°C and incubation was continued for 3 h (lanes 2, 4, 6, and 8). Molecular mass is expressed in kilodaltons.

higher ELISA titers to the surface polysaccharides and proteins and the HP than did immunization with the A-1 tight mutant ($P < 0.05$). In contrast, the 12-1 coaster mutant induced significantly higher ELISA titers only against the surface polysaccharides ($P < 0.05$). Challenge with the WT induced significantly higher serum ELISA titers to the surface polysaccharides and proteins compared with titers induced by immunization ($P < 0.05$); the anti-HP ELISA titers of sera from mice immunized with the A-1 tight and 4-1 coaster mutants did not, however, significantly increase after challenge with the WT.

WT *A. pleuropneumoniae* was recovered from the lungs of all the mice that died after challenge, but by 14 days after challenge no bacteria were recovered from the lungs of any of the mice that survived.

DISCUSSION

A. pleuropneumoniae is a major cause of morbidity and mortality in swine herds (28); surviving pigs are often stunted and

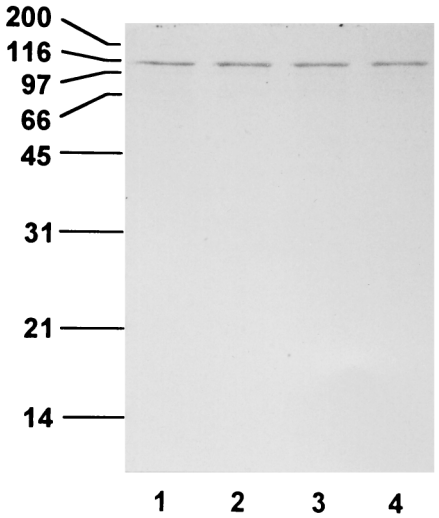

FIG. 3. Coomassie blue-stained SDS-polyacrylamide gel of HP purified from WT and TS mutant *A. pleuropneumoniae* 4074. The HPs, purified as described in Materials and Methods, were applied to a 12% polyacrylamide gel (9 μg of protein in 20 μl per lane). Lane 1, WT; lane 2, TS mutant A-1; lane 3, 4-1; lane 4, 12-1. Molecular mass is expressed in kilodaltons. A band with a molecular mass of 105 kDa was detected in all the samples.

TABLE 2. Cumulative mortality of immunized and control mice after challenge with *A. pleuropneumoniae*[a]

Time (h)	No. of mice immunized with[b]: Control (n = 17)		A-1 (T) (n = 14)		4-1 (C) (n = 18)		12-1 (C) (n = 17)	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
12	6	11	10	4	17	1	17	0
24	3	14	9	5	15	3	16	1
48	2	15	8	6	12	6	16	1
72	0	17	8	6	12	6	14	3
Total (% survival)	0 (0)		8 (57*)		12 (67*)		14 (82*)	

[a] Groups of female ICR mice were immunized three times i.n. with TS mutants or saline and subsequently challenged with the WT as described in Materials and Methods. The data were combined from two separate experiments.
[b] T, tight mutant; C, coasting mutant; *, significantly different from the control group ($P < 0.05$).

frequently become asymptomatic carriers of the organism (24, 27). Swine pleuropneumonia is currently one of the most costly diseases affecting the swine industry worldwide, with losses in the millions of dollars.

Whole-cell bacterins have been used to help control SPAP; they induce antibodies only against cell wall components, such as the CP, LPS, and OMP, not against secreted HP, and give a very limited serotype-specific protection (8, 13, 26). A live attenuated mutant that is nonvirulent in pigs but maintains the virulence factors of the parental WT would make an excellent vaccine candidate.

Temperature sensitivity as a form of genetic attenuation gives several advantages over other types of attenuation (7, 11, 12, 14, 21–23). First, TS mutants with lesions in genes that encode essential proteins cannot grow at the nonpermissive temperature in any nutritional environment, as the effect of the mutation cannot be reversed by exogenous nutrient supplementation. Second, surface antigens are present and remain intact in their native configuration, so that their immunogenicity is uncompromised. Third, immunization with TS mutants could allow limited production of exotoxin(s) against which the host can produce antibodies. Fourth, TS mutants that have limited replication in the vaccinee can be readily isolated; immunization with these mutants may mimic the early stages of natural infection, allowing a more prolonged stimulation of the host immune system and the expression of genes coding for antigens that are synthesized only in vivo. Finally, individual mutations (each with a reversion frequency of ca. 10^{-7}) can be combined in one strain to reduce the frequency of reversion to virulence of the vaccine strain to negligible levels (ca. 10^{-21}), ensuring safety (21, 22).

The production of hemolysin is critical for the virulence of *A. pleuropneumoniae* and for inducing immunoprotection in the host. A nonhemolytic mutant of a serotype 5 strain of *A. pleuropneumoniae* isolated after nitrosoguanidine mutagenesis lacked the 105-kDa HP but maintained a full parental complement of capsular, LPS, and outer membrane components (15). The mutant did not induce death or lesions in mice or pigs challenged with large doses, but immunization of mice and pigs with the mutant did not provide protection against challenge with the parental strain. Thus, it was vital that the TS mutant strains that we isolated maintain the ability to synthesize and secrete the HP after immunization to induce an immune response. Moreover, Western blotting has established that there is cross-reactivity among the exotoxins (105 to 109 and 120 kDa) from different strains and serotypes of *A. pleuropneumoniae* (9, 10), and inclusion of the HP in a vaccine could induce protection not only against strains within the same serotype but also against strains of different serotypes.

Treatment with nitrosoguanidine often produces mutations at multiple sites within the chromosome, so biochemical and physical parameters were evaluated to detect any major phenotype alterations in the TS mutants. There were no major differences between the TS mutants and the WT in their biochemical and agglutination reactions; electrophoretic LPS, OMP, and HP profiles (Fig. 2 and 3); hemolytic titers at 30°C; and CAMP factor reactions.

i.n. immunization with both the tight and coaster mutants elicited significant levels of protection in mice against challenge with the WT, with one of the coaster strains (12-1) inducing the highest level (82%) (Table 2). Immunization with the TS mutants also induced serum ELISA antibodies against the surface polysaccharides and proteins and against the HP. It is significant to note that the coaster mutants (4-1 and 12-1) eliciting the highest level of protection likewise induced the highest level of anti-HP antibodies both postimmunization and postchallenge. It is clear that all three mutants produced enough HP in vivo for the induction of antibodies even though HP activity could not be detected at the nonpermissive temperature in vitro.

Our results confirm and, in at least one respect, extend the results of Inzana et al. (14). Those workers isolated TS mutants of *A. pleuropneumoniae*, albeit by a different method, and tested only one of them in a very limited number of mice and

TABLE 3. Serum ELISA antibody responses of immunized and challenged mice to polysaccharide and protein antigens of *A. pleuropneumoniae*[a]

TS mutant[c]	Titer (range)[b]: Postimmunization CP	LPS	OMP	HP	Postchallenge CP	LPS	OMP	HP
A-1 (9; 3)	2.4 (2.2–2.8)	2.4 (2.2–2.5)	2.7 (2.5–2.8)	2.8 (2.8–3.1)	3.7 (3.2–3.9)	3.8 (3.4–4.0)	3.6 (3.4–3.7)	2.9** (2.8–3.0)
4-1 (18; 12)	3.3* (3.0–3.5)	3.2* (2.9–3.4)	3.1* (2.7–3.5)	3.4* (3.2–3.5)	3.9 (2.8–4.2)	4.0 (3.0–4.2)	4.0 (2.6–4.4)	3.6** (2.6–4.1)
12-1 (17; 14)	3.1* (2.8–3.1)	3.1* (2.5–3.2)	3.1 (2.2–3.6)	3.1 (2.1–3.5)	3.9 (3.0–4.2)	4.0 (2.7–4.2)	3.7 (2.9–4.0)	3.7 (2.5–4.1)

[a] Sera were collected 14 days after final immunization with the TS mutants and 14 days after challenge with wild-type *A. pleuropneumoniae* 4074.
[b] ELISA titers are expressed as the mean $\log_{10}$ values of the reciprocal of the highest dilution at which the absorbance was at least twice that of the nonimmune serum at the same dilution. The mean A_{410} values for the nonimmune, control sera for each antigen for both postvaccination and postchallenge samples, respectively, were as follows: CP, 0.05 and 0.03; LPS, 0.05 and 0.03; OMP, 0.03 and 0.03; and HP, 0.05 and 0.03. *, Significantly different from titers induced by the A-1 TS mutant ($P < 0.05$); **, not significantly different from the HP postimmunization titers induced by the A-1 and 4-1 TS mutants. All other titers in the postchallenge groups were significantly different from the postimmunization titers ($P < 0.05$).
[c] The first number in parentheses is the number in the postimmunization group; the second number is the number in the postchallenge group.

pigs. One significant difference between their results and ours is that the TS mutants that we isolated were considerably diminished in virulence. The LD_{50} of each of our TS mutants for mice was at least half a log unit higher than that of the WT (data not shown); the LD_{50}s for mice of the WT and the TS mutant described by Inzana et al. were identical (14). The second difference is that our TS mutants are apparently much more stable than those isolated by Inzana et al. (14). Several of their mutants reverted to the WT after a few passages, whereas our TS mutants have maintained their phenotype after repeated passages. The reason for these differences in reversion rates is not immediately obvious. Nevertheless, the reversion frequencies of our TS mutants are still too high for immediate application as vaccine candidates. A genetically stable vaccine strain will have to be constructed from independent TS mutations, and the studies required for the construction of the strain are currently under way. Further studies are also needed to determine the efficacy of immunization with the TS mutants in pigs.

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